Functional Genomics of Eukaryotic Photosynthesis Using Insertional Mutagenesis of *Chlamydomonas reinhardtii*[1]

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The unicellular green alga *Chlamydomonas reinhardtii* is a widely used model organism for studies of oxygenic photosynthesis in eukaryotes. Here we describe the development of a resource for functional genomics of photosynthesis using insertional mutagenesis of the Chlamydomonas nuclear genome. Chlamydomonas cells were transformed with either of two plasmids conferring zeocin resistance, and insertional mutants were selected in the dark on acetate-containing medium to recover light-sensitive and nonphotosynthetic mutants. The population of insertional mutants was subjected to a battery of primary and secondary phenotypic screens to identify photosynthesis-related mutants that were pigment deficient, light sensitive, nonphotosynthetic, or hypersensitive to reactive oxygen species. Approximately 9% of the insertional mutants exhibited 1 or more of these phenotypes. Molecular analysis showed that each mutant line contains an average of 1.4 insertions, and genetic analysis indicated that approximately 50% of the mutations are tagged by the transforming DNA. Flanking DNA was isolated from the mutants, and sequence data for the insertion sites in 50 mutants are presented and discussed.

As with other model organisms, the availability of genome sequence data is revolutionizing and revitalizing research into the biology of the unicellular green alga *Chlamydomonas reinhardtii* (Grossman et al., 2003; Ledford et al., 2005). Over the past four decades, many fundamental insights into the structure, function, assembly, and regulation of the photosynthetic apparatus have come from studies of Chlamydomonas, which offer several advantages for the genetic dissection of eukaryotic photosynthesis (for review, see Davies and Grossman, 1998; Hippler et al., 1998; Grossman, 2000; Dent et al., 2001; Rochaix, 2001). First and foremost, photosynthesis is fully dispensable in Chlamydomonas, as cells can grow heterotrophically in the dark using acetate as a sole carbon source. Cells grown in the dark, however, still synthesize and assemble a fully functional photosynthetic apparatus. This allows the isolation and analysis of mutants that are unable to perform photosynthesis, and light-sensitive mutants can be maintained in complete darkness. Because Chlamydomonas is predominantly maintained in a haploid form, it is not necessary to generate homozygous nuclear mutants, and mutants affecting photosynthesis can be screened immediately following mutagenesis. Chlamydomonas has an easily controlled and rapid sexual cycle (approximately 2 weeks) with the possibility of tetrad analysis, which facilitates genetic analysis. Its rapid cell-doubling time (approximately 10 h) and microbial lifestyle mean that it is easy to grow homogeneous cultures on any scale, simplifying physiological and biochemical characterization in comparison to multicellular land plants (Ledford et al., 2005). By way of example, the application of inhibitors and generators of various types of reactive oxygen species results in uniform uptake of the chemical by each cell. In land plants, multicellularity leads to differential uptake of exogenous substances based upon the distance from or method of application, and different tissue and cell types may react differently to any given chemical, making analysis of results difficult.

In spite of these differences, however, the photosynthetic apparatus of Chlamydomonas is very similar to that of land plants, making it a useful comparative system for understanding plant metabolism and photosynthesis (Gutman and Niyogi, 2004). As a member of the division Chlorophyta, Chlamydomonas is also a useful model for investigating evolutionary relationships among the green algae and thus the origins of photosynthesis in land plants.

The first draft of the Chlamydomonas nuclear genome sequence was released in January, 2003 (Grossman et al., 2003), and a complete, fully annotated version is expected in the near future. The recent accumulation of expressed sequence tag (EST) sequence data (Asamizu et al., 1999; Shrager et al., 2003) has both facilitated annotation and given some indication of the degree of accuracy that can be achieved when using bioinformatic tools to predict gene structure from assembled sequence data in this organism. The completion of the genome sequences of *Volvox carteri* and *Ostreococcus tauri* will also aid in this endeavor to identify the complete gene set of Chlamydomonas.

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Now that the sequencing phase of Chlamydomonas genomics is nearing completion, the next step is the functional characterization of the genes. Sequence comparison and phylogenetic approaches can be used to identify putative functional homologs of genes whose functions are known in other organisms, but mutagenesis is one of the most powerful methods for assigning function to a given gene or gene family. In Chlamydomonas, insertional mutagenesis has proved to be a very useful tool in forward genetics studies, which aim to identify genes involved in a given process. Integration of exogenous DNA into the nuclear genome of Chlamydomonas occurs predominantly by nonhomologous recombination, thus leading to random gene disruption (Tam and Lefebvre, 1993). In most cases, insertional mutagenesis creates null mutations. In comparison to point mutations, insertional mutagenesis allows the isolation of sequence flanking the mutation by methods such as plasmid rescue and PCR-based techniques. Although the recent development of a detailed molecular map (Kathir et al., 2003) has made the mapping of point mutations relatively rapid in Chlamydomonas, this is still not a viable alternative for high-throughput analysis of large numbers of mutants.

Although insertional mutagenesis has been used extensively in the investigation of many areas of Chlamydomonas biology, only one study has described the use of the technique at a genomics level. Pazour and Witman (2000) reported the use of a genomic approach, involving both forward and reverse genetics, to isolate mutations affecting the outer dynein arm of Chlamydomonas flagella. This structure consists of a total of 15 proteins, thus giving some indication of the number of target genes that were involved. Mutations in the outer dynein arm result in a characteristic slow, jerky, swimming phenotype. After screening 15,000 insertional mutants for this phenotype, mutations in 7 of the 15 target genes were identified.

The paucity of studies at the genome level illustrates the need for more extensive functional genomic analyses and resources for Chlamydomonas to complement the already considerable sequence information that is available. The generation of large mutant collections has been vital in the development and use of other model plant systems such as Arabidopsis (*Arabidopsis thaliana*; Krysan et al., 1999; Tissier et al., 1999; Parinov and Sundaresan, 2000; McElver et al., 2001; Sessions et al., 2002; Alonso et al., 2003), rice (*Oryza sativa*; Jeon et al., 2000; Chen et al., 2003; Kolesnik et al., 2004; Sallaud et al., 2004), and maize (*Zea mays*; Raizada et al., 2001; May et al., 2003). Therefore, we have initiated a large-scale forward genetics project using insertional mutagenesis that aims to saturate the Chlamydomonas nuclear genome for mutations affecting photosynthesis as part of the Chlamydomonas Genome Project (Grossman et al., 2003). In this article, we describe the mutant generation and screening methods being employed in this project. As a resource to workers in the field who will be using these mutants, the phenotypic, molecular, and genetic characteristics of a subset of mutants are reported here, in addition to flanking sequence data. The whole population of phenotypically characterized mutants and a searchable sequence database will be available to the scientific community as they are generated over the next several years.

RESULTS

Generation of Insertional Mutants

To isolate insertional mutants affecting all aspects of photosynthesis in Chlamydomonas, selection of transformed cells in the dark was necessary. Although mutants incapable of photoautotrophic growth can be isolated and maintained as acetate-requiring mutants in the light, this approach does not allow the recovery of all photosynthetic mutants (Spreitzer and Mets, 1981). Very few mutants with defects in the CO_2 fixation reactions of photosynthesis, for example, can be recovered this way, because the mutants are light sensitive.

After comparison of the growth of several wild-type Chlamydomonas strains in the dark, the strain 4A+ in the 137c genetic background was selected as the parental strain for the population of insertional mutants based on its ability to grow well and remain green in the dark. Cells were transformed with either of 2 linearized plasmids, pSP124S or pMS188 (Fig. 1), containing the *ble* gene, which confers resistance to the antibiotic zeocin (bleomycin), and transformants were selected on acetate-containing medium in the dark. Transformation efficiencies using the 4A+ strain were 86.5 transformants/μg DNA for pSP124S and 115.5 transformants/μg DNA for pMS188. Both of these efficiencies are lower than those reported for these plasmids in other studies (Lumbreras et al., 1998; Schroda et al., 2002), suggesting that 4A+ may transform at lower efficiencies than cell wall-deficient strains and other strains that were used previously. Here we report data for a total of 2,000 insertional mutants generated using pSP124S and 760 using pMS188.

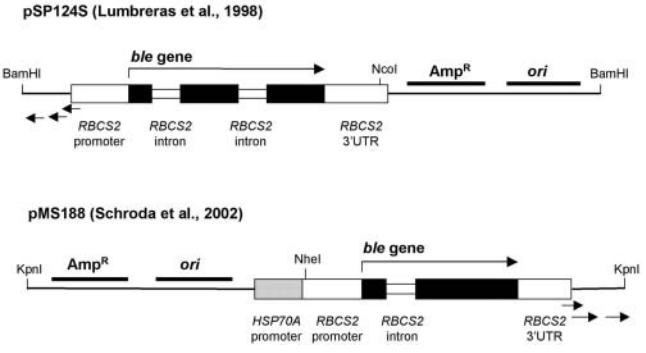

Figure 1. Diagram of linearized plasmids used for insertional mutagenesis. Relevant restriction enzyme sites are shown. Arrows indicate the approximate positions of specific primers used for TAIL-PCR.

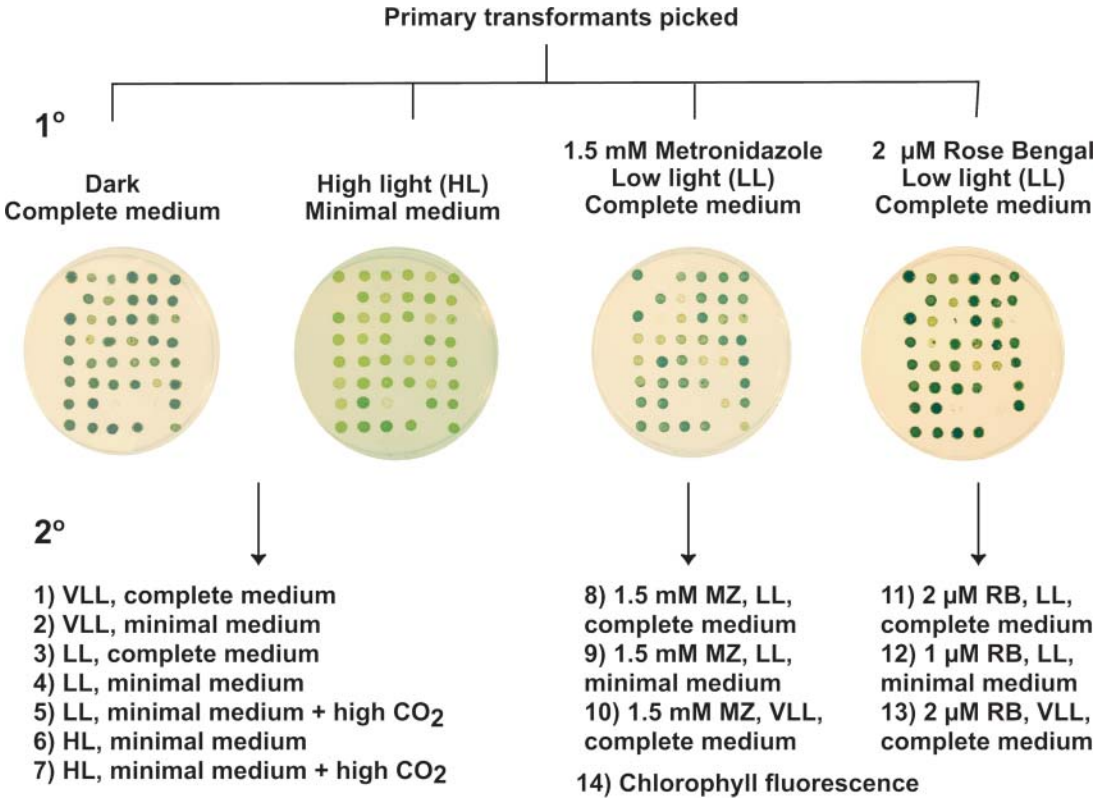

Figure 2. Outline of primary and secondary screening procedures for the isolation of mutants with photosynthesis-related phenotypes. VLL, 3 μmol photons m^{-2} s^{-1}; LL, 80 μmol photons m^{-2} s^{-1}; HL, 500 μmol photons m^{-2} s^{-1}.

Results of Phenotypic Screening

Insertional mutants were subjected to primary and secondary rounds of phenotypic screening (Fig. 2). The primary screens included incubation of the mutants at high light (HL; 500 μmol photons m^{-2} s^{-1}) on minimal medium to isolate all light-sensitive or nonphotosynthetic clones. Two generators of reactive oxygen species were used to isolate mutants that are sensitive to photooxidative stress, which often accompanies photosynthesis. Like chlorophyll, Rose Bengal (RB) generates singlet oxygen in the presence of light. By growing cells on medium containing RB, elevated levels of singlet oxygen would be present within cells and in the surrounding medium. Metronidazole (MZ), however, acts by accepting electrons from reduced ferredoxin and catalyzing superoxide formation in the chloroplast compartment of Chlamydomonas (Schmidt et al., 1977). The secondary screening methods were designed to characterize the phenotype of primary mutants more fully by assessing the degree of light sensitivity (at various light intensities) and ascertaining whether the response to generators of reactive oxygen species was dependent on photoautotrophic or heterotrophic growth conditions (Fig. 2).

The proportions of mutants in each major phenotypic class are presented in Table I. The total proportion of mutants showing a phenotype in any of the screens was 8.8%. It should be noted that the classes of mutants presented in Table I are not mutually exclusive, and thus mutants may show a phenotype in more than one of the test screens.

The largest class of mutants recovered was the acetate-requiring mutants. In agreement with Spreitzer and Mets (1981), most of these also exhibited some sensitivity to light, either at the low-light (LL; 80 μmol photons m^{-2} s^{-1}) or HL level. Secondary screening showed that 18% of the acetate-requiring mutants could be rescued, at least partially, under conditions of high CO_2. The LL-, HL-, RB-, and MZ-sensitive classes all occurred at a frequency of approximately 2.3%. Of the total number of mutants found to be sensitive to either generator of reactive oxygen species, only one-third showed sensitivity to both RB and MZ. The smallest mutant class comprised the pigment-deficient mutants, and these occurred at a frequency of 0.6%. This class included mutants that were pale green in all

Table I. *Percentage of mutants in each phenotypic class*

Phenotype	Percentage
LL sensitive (≥80 μmol photons m^{-2} s^{-1})	2.3
Acetate requiring	3.8
HL sensitive (≥500 μmol photons m^{-2} s^{-1})	2.3
RB sensitive	2.3
MZ sensitive	2.3
Pigment deficient	0.6

treatments, or white, yellow, or brown in at least one treatment.

Molecular Analysis of Transformants

To characterize the average number of *ble* insertion loci in each mutant, DNA gel-blot analysis was carried out on those mutants that exhibited a phenotype in any of the screens. For the population generated using the pSP124S plasmid, 85 mutants were analyzed, and 30 were analyzed for which pMS188 was the transforming plasmid. Figure 3 shows examples of the DNA gel-blot analysis. It was found that, for both plasmids, approximately 70% of the transformants contained a single *ble* insertion locus (61/85 for pSP124S and 22/29 for pMS188). The average number of *ble* insertion loci for pSP124S was 1.4 and for pMS188 it was 1.3. It should be noted that this analysis would not be able to identify clones in which multiple *ble* insertions had occurred at one locus.

In addition to probing for the sequence encoding the *ble* gene, 53 of the mutants were also analyzed for the presence of the origin of replication from the pBluescript portion of the transforming plasmid. Thirty-one of the 53 mutants (58.5%) were found to have 1 or more bands hybridizing to this sequence. Of these 31, however, 23 (74%) contained bands of the same size that hybridized to both the *ble* probe and the origin of replication probe. Because the genomic DNA was digested with *Nco*I, which should cut between these sequences in the linearized transforming plasmid (Fig. 1), bands of different sizes should be detected with the two probes. This suggests that the clones in which the same-size fragment was detected all contained tandem head-to-tail insertions at the same locus.

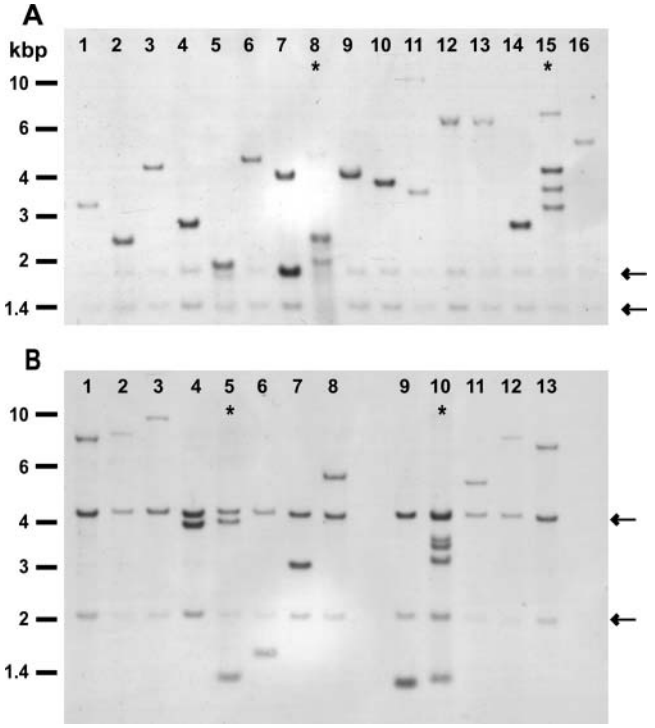

Figure 3. DNA gel-blot analysis of insertional mutants. Arrows indicate bands corresponding to endogenous *RBCS2* sequences, and asterisks indicate mutants containing multiple *ble* insertions. Size standards are shown to the left. A, Mutants generated using pSP124S. Genomic DNA was digested with *Nco*I, and the probe was a *Xba*I/*Bam*HI fragment from pSP124S. B, Mutants generated using pMS188. Genomic DNA was digested with *Nhe*I, and the probe was a *Nhe*I/*Kpn*I fragment from pMS188.

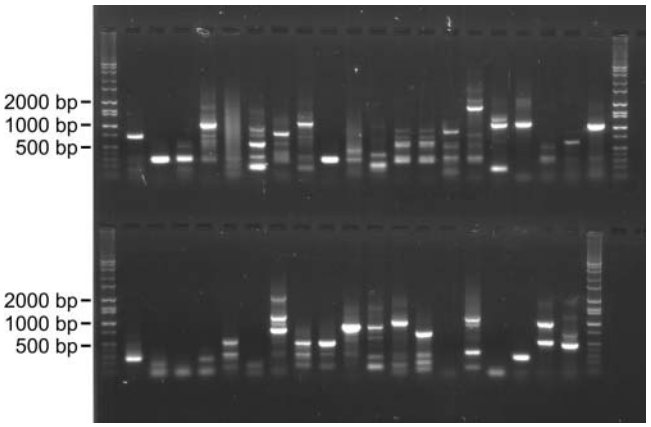

Figure 4. Agarose gel analysis of tertiary TAIL-PCR products from 39 insertional lines. Size standards are shown to the left.

Isolation and Sequencing of Flanking DNA

After secondary screening, DNA was extracted from all mutants that rescreened with the same phenotype as recorded in the primary screen. Flanking DNA was amplified from each insertional mutant line using thermal asymmetric interlaced (TAIL)-PCR (Liu et al., 1995). At least 1 DNA band was amplified in 77% of mutants where pSP124S was used as the transforming plasmid. Figure 4 shows a representative agarose gel analysis of fragments amplified from a subset of insertional mutants. The size of bands amplified using this technique ranged from <100 to 2,000 bp, with most bands being in the 100- to 1,000-bp range. Single bands were amplified in 44% of the mutants tested.

One of the problems encountered with the TAIL-PCR technique is that some of the insertion lines contained concatameric insertion events at a single locus. As insertion events included tandem arrays of the transforming DNA, sequencing of the product from TAIL-PCR only yielded plasmid sequence. For pSP124S, many of these mutants could easily be recognized by a diagnostic band of 750 bp, and several other DNA fragments also yielded only plasmid sequence. Overall, in 15.3% of the mutants from which a TAIL-PCR product was amplified, it was not possible to obtain the flanking DNA sequence due to concatamerization at the site of insertion.

Table II presents the flanking sequence results for the fragments generated by TAIL-PCR from 50 mutants. Sequences were compared to the Chlamydomonas

Table II. *Phenotypic description, flanking sequence data (by similarity to the Chlamydomonas genome sequence), and results of molecular analysis of insertional mutants*

Mutant ID	Phenotype(s)	Genome Position and Candidate Gene(s)[a]	No. of *ble* Insertions[b]
CAL005.01.01	Bleaches on HS	**185:** 23950–23500	n.d.
CAL005.01.13	Acetate requiring, LL sensitive	**248:** 49881–50798 Genie 248.7 *RBCS1* (Chlamydomonas) Genie 248.8 *RBCS2* (Chlamydomonas)	1
CAL005.01.20	RB sensitive	**1725:** 13500–13889	1
CAL005.01.21	LL sensitive, acetate requiring	**2187:** 552–627	1
CAL005.01.26	LL sensitive, acetate requiring	**248:** 14045–13965	1
CAL007.01.01	Bleaches at LL, RB, and MZ sensitive, low chlorophyll fluorescence	**86:** 72908–73019 Genie 86.13: β-7 subunit of 20S proteasome (rice) Genie 86.14: Histone-binding protein N1/N2 (*Xenopus laevis*)	1
CAL007.01.04	RB sensitive	**1243:** 2407–2199 Genie 1243.1 and 1243.2: Trans-splicing factor *Raa3* (Chlamydomonas)	1
CAL007.01.09	Yellow in the dark and at VLL	**137:** 48662–48363 Genewise 137.14.1: *crtH*; carotene isomerase (*Synechocystis* sp. PCC6803)	2
CAL007.01.11	Bleaches on HS, MZ sensitive	**125:** 47270–46956 Genewise 125.48.1: *hemD*, uroporphyrin III-synthase (*Synechocystis* sp. PCC6803)	
CAL007.01.17	Bleaches on MZ/HS	**45:** 114609–114355	1
CAL007.01.18	Slight RB sensitivity	**876:** 16393–16434 Genie 876.2: Acetyl CoA synthetase (Arabidopsis)	1
CAL007.01.24	LL sensitive, acetate requiring, high chlorophyll fluorescence	No genome similarity	1
CAL007.01.25	Slight RB sensitivity	**1543:** 7155–6541	1
CAL007.01.26	Acetate requiring, MZ sensitive	**416:** 20998–20655	2
CAL007.01.29	Slight acetate requirement	No genome similarity Identity to EST: 1031030D08.y1	1
CAL007.01.30	HL sensitive, acetate requiring, MZ and RB sensitive	Multiple hits, repeat region	2
CAL007.01.40	RB sensitive	**387:** 43424–43184	1
CAL007.01.42	RB sensitive, slight MZ sensitivity	**785:** 20041–19556 Genie 785.4: *HSP101* (Arabidopsis)	1
CAL007.01.43	MZ sensitive, some RB sensitivity	**239:** 60731–60473 Genie 239.1.1 Histone H2A (Chlamydomonas) Genie 239.37.1 Histone H3 (*Volvox carteri*) Genie 239.7.1 Histone H4 (Chlamydomonas)	1
CAL007.01.46	Slight RB sensitivity	**119:** 81484–81721	3
CAL007.02.02	LL sensitive, acetate requiring, partially rescued by high-CO_2, low-chlorophyll content	**595:** 18007–17767	1
CAL007.02.03	LL sensitive, acetate requiring	**1152:** 5548–5360 Genie 1152.2 ATP-synthase δ-chain (Chlamydomonas)	3-5
CAL007.02.09	RB sensitive, reduced pigment at high CO_2	**91:** 50935–50800 Genie 91.10: ODA1 outer dynein arm docking protein (Chlamydomonas) Genie 91.11 Silencing-related Ser-Thr kinase (rice)	1
CAL007.02.10	MZ sensitive	**563:** 15107–14707 Genie 563.3 Digalactosyldiacylglycerol synthase (Arabidopsis)	1
CAL007.02.18	Slight RB and MZ sensitivity	**1535:** 12114–12248	n.d.
CAL007.02.19	Slight RB and MZ sensitivity	**851:** 9886–9587 Genewise 851.5.1: Putative Cu(II)-type ascorbate-dependent monooxygenase (Arabidopsis)	n.d.
CAL007.02.21	Acetate requiring, HL sensitive, rescued by high CO_2, RB sensitive, high chlorophyll fluorescence	Multiple hits in genome, repeat region	1

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

Mutant ID	Phenotype(s)	Genome Position and Candidate Gene(s)[a]	No. of *ble* Insertions[b]
CAL007.02.27	MZ sensitive, low growth rate	**89:** 98986–99143 Genie 89.16: Potential Cu-transporting ATPase type 3 (Arabidopsis) Genie 89.17: Glutathione-requiring prostaglandin D-synthase (*Gallus gallus*)	1
CAL007.02.31	Bleaches on MZ/HS	**65:** 19631–20008 Genie 65.2: Putative replication factor (Arabidopsis)	3
CAL007.02.38	Acetate requiring	**1700:** 10826–11047 Genie 1700.6: Putative NADP oxidase (*Vibrio cholerae*) Genie 1700.2–1700.5 Histone cluster (H3, H4, H2A, H2B-IV) Genie 1700.1: Phosphoglycolate phosphatase chloroplast precursor (Chlamydomonas)	1
CAL007.02.47	Slight MZ sensitivity	**68:** 17490–17867 Genie 68.3: Protein phosphatase 2C ABI1 (Arabidopsis)	2
CAL007.03.02	RB sensitive	**1380:** 4193–4169	2
CAL007.03.03	HL sensitive, RB sensitive	Multiple hits in genome, repeat region	1
CAL007.03.08	Slight MZ sensitivity	**2640:** 5102–5626 Genie 2640.0: 70-kD heat shock protein (Chlamydomonas)	2
CAL007.03.10	MZ sensitive	Multiple hits in genome, repeat region	2
CAL007.03.21	Acetate requiring	**590:** 28275–27677 Genie 590.2 and 590.3: repair endonuclease (Arabidopsis)	1
CAL007.03.22	MZ sensitive, slight RB sensitivity	**276:** 20397–20609 Genewise.276.32.1 Dynein 11-kD light chain flagellar outer arm (Chlamydomonas) Genie 276.2 cgcr-4 protein (Chlamydomonas)	1
CAL007.03.26	MZ sensitive	Fragment 1: multiple hits, repeat region Fragment 2: **228:** 4384–4683	1
CAL007.03.32	Acetate requiring, HL sensitive, rescued by high CO_2	**3868:** 836–879	2
CAL007.03.34	HL sensitive, partially acetate requiring, rescued by high CO_2	**899:** 9585–10323	3
CAL007.03.41	Acetate requiring, RB sensitive	Fragment 1: **199:** 124–58 Genie 199.1: phospho*enol*pyruvate-dependent sugar phosphotransferase system Fragment 2: **901:** 31035–30595	4
CAL007.03.43	MZ sensitive	**248:** 42663–48811 (discontinuous) Genie 248.8: *RBCS2*	1
CAL007.03.45	Acetate requiring	**3:** 156677–156894	2
CAL007.03.46	Acetate requiring, partially rescued by high CO_2	Multiple hits, repeat region	2
CAL007.03.47	Acetate requiring, HL sensitive	**45:** 151884–152295	1
CAL010.01.02	RB sensitive	**732:** 12958–12763 Genie 732.2 Autolysin (gametolysin) precursor (Chlamydomonas)	1
CAL010.01.10	RB sensitive, pale green	**1214:** 1678–1021 (discontinuous)	1
CAL010.01.11	LL sensitive	**62:** 19180–19068 Genie 62.4 Genie 62.5 Succinate dehydrogenase (ubiquinone) iron-sulfur protein precursor (Drosophila)	1
CAL010.01.21	RB sensitive, MZ sensitive	**23:** 50197–50354	1
CAL010.01.31	RB sensitive	**102:** 2403–2506 Genewise 102.30.1: Calmodulin-binding protein (Arabidopsis)	n.d.

[a]Determined by comparison with the Chlamydomonas nuclear genome sequence, version 1.0 (http://genome.jgi-psf.org/chlre1/chlre1.home.html). Alignment of the flanking sequence with the genome sequence is indicated by scaffold number (in bold) followed by sequence range in base pairs. [b]Number of *ble* insertions determined by DNA gel-blot analysis. n.d., Not determined.

genome sequence (version 1.0; http://genome.jgi-psf.org/chlre1/chlre1.home.html) and to Chlamydomonas ESTs if no genome similarity was found. Of the 50 mutants presented, only 2 did not show similarity to any region in the genome sequence, and 1 of these showed similarity to an EST sequence. Because the integration of transforming DNA in the Chlamydomonas nucleus is sometimes accompanied by a deletion at the site of insertion, candidate genes in Table II were identified based on gene models that occur within a 10-kb interval beginning at the insertion site and extending in the direction of the *ble* insert. The identification of candidate genes was limited somewhat by incomplete assembly and annotation of the genome.

Nevertheless, likely candidate genes could be identified for several mutants (Table II). In the acetate-requiring phenotypic class, putative mutants were isolated in the Rubisco small subunit (*RBCS*) locus (see below), the ATP synthase δ-subunit gene (CAL007.02.03), and a gene involved in the phospho*enol*pyruvate-dependent sugar phosphotransferase system (CAL007.03.41). In the pigment-deficient mutant class, CAL007.01.09, which is yellow in the dark, was shown to have an insertion in a gene exhibiting homology to the *Synechocystis* sp. PCC 6803 carotene isomerase or *crtH* gene. Among mutants that are sensitive to RB and/or MZ, candidate genes include 2 heat shock protein genes, *HSP101* (CAL007.01.42) and *HSP70A* (CAL007.03.08), a putative sigma-class glutathione *S*-transferase gene (CAL007.02.27), a putative digalactosyldiacylglycerol synthase gene (CAL007.02.10), and a putative Cu(II)-type ascorbate-dependent monooxygenase gene (CAL007.02.19). Mutant CAL007.01.17 has an insertion downstream from a gene showing homology to the uroporphyrin III-synthase gene (*hemD*) from Synechocystis. This mutant is sensitive to MZ and bleaches on minimal medium in HL, suggesting that siroheme and/or vitamin B12 may be involved in the response to superoxide and photooxidative stress.

Interestingly, several mutants were found to have insertions at or close to the *RBCS* locus, which contains the *RBCS1* and *RBCS2* genes. For pSP124S (111 sequences in total), this was found in 3 independent mutants (CAL005.01.13, CAL005.01.26, and CAL007.03.43). The pSP124S plasmid contains promoter, 3′-untranslated region, and intron sequences from *RBCS2* (Fig. 2). The lines CAL005.01.13 and CAL005.01.26 both have a light-sensitive, acetate-requiring phenotype consistent with a deletion of both *RBCS* genes (Khrebtukova and Spreitzer, 1996). Genetic analysis showed that CAL005.01.13, reported previously as *dim1* (Dent et al., 2001), is tagged by the transforming DNA (Table III). Isolation of the flanking sequence from both sides of the insert by plasmid rescue showed that a deletion of approximately 36 kb of genomic DNA has occurred in CAL005.01.13, and this deletion affects the entire *RBCS* locus (Dent et al., 2001). Subsequent work with this mutant has shown that the phenotype can be rescued by complementation with either the *RBCS1* or *RBCS2* genes (R.J. Spreitzer, personal communication). The flanking sequence from CAL007.03.43 showed the insertion to be immediately downstream of the *RBCS1* gene, suggesting that the *RBCS* locus is intact in this mutant. Consistent with this analysis, the mutant does not have an acetate-requiring or light-sensitive phenotype, although it is MZ sensitive (Table II).

Table III. *Genetic analysis of insertional mutants*

Mutant ID	Recombinants[a]/Total Progeny		Linkage
	Progeny from Complete Tetrads	Progeny from Incomplete Tetrads	
CAL005.01.13	0/16	0/25	Yes
CAL005.01.15	11/16	14/23	No
CAL005.01.16	0/144	–	Yes
CAL005.01.21	0/20	0/43	Yes
CAL005.01.26	0/48	–	Yes
CAL005.01.28	0/16	0/31	Yes
CAL007.01.01	6/12	8/13	No
CAL007.01.08	0/40	0/15	Yes
CAL007.01.09	0/16	0/41	Yes
CAL007.01.13	2/8	4/26	No
CAL007.01.20	–	8/27	No
CAL007.01.24	9/36	–	No
CAL007.01.30	0/36	0/28	Yes
CAL007.01.39	18/40	–	No
CAL007.02.02	0/48	–	Yes
CAL007.02.05	14/44	–	No
CAL007.02.38	18/44	10/25	No

[a]Recombinants include zeocin-sensitive progeny that have the screened phenotype and zeocin-resistant progeny that lack the screened phenotype. Dash indicates no progeny of that type.

Genetic Analysis

To analyze the frequency with which the mutation is linked to the transforming DNA in the population of screened mutants, several mutants were crossed to an *mt*− wild-type strain. The progeny were then analyzed for cosegregation of the zeocin-resistance phenotype with the phenotype characterized during the screening procedure. Table III shows the linkage results of 17 crosses. A total of nine mutants (52%) showed no recombinant progeny, demonstrating linkage of the screened phenotype to the transforming DNA. With the number of progeny analyzed and assuming an average of 100 kb/cM in Chlamydomonas (Kathir et al., 2003), the transforming DNA would be inserted within 50 to 100 kb of the gene or genes resulting in the screened phenotype. More progeny would need to be analyzed to state with certainty that the mutation is indeed tagged.

DISCUSSION

The last 10 years have heralded the sequencing era in biology. As more and more genome sequences

become available, one of the most significant findings being revealed is the large number of genes for which no function is known or can be predicted by sequence similarity alone. Inactivation of a gene is generally the most direct way to understand its function. An essential tool for the functional analysis of sequenced genomes is therefore the ability to create loss-of-function mutations for all of the genes (Alonso et al., 2003). Thus far, this has only been achieved for the unicellular budding yeast *Saccharomyces cerevisiae* (Giaever et al., 2002), utilizing targeted gene replacement via homologous recombination. Unfortunately, this tool is not available in many eukaryotic organisms. Gene silencing has recently been employed to study the role of approximately 86% of the predicted genes in the *Caenorhabditis elegans* genome (Kamath et al., 2003). However, RNA interference-based methods of gene inactivation have several drawbacks, including the lack of stable heritability of a phenotype and variable levels of residual gene activity. For organisms in which homologous recombination is not available, therefore, libraries of sequence-indexed insertional mutants have many advantages (Parinov and Sundaresan, 2000). Although insertional mutagenesis has been used successfully in the generation of mutant libraries in animals (Kaiser and Goodwin, 1990; Zwaal et al., 1993; Golling et al., 2002), their strength has been most convincingly demonstrated in plants (Alonso et al., 2003). Large mutant collections exist for both T-DNA and transposon insertional lines in Arabidopsis (Sundaresan et al., 1995; Tissier et al., 1999; Sessions et al., 2002; Alonso et al., 2003), maize (May et al., 2003), and rice (Kim et al., 2004; Kolesnik et al., 2004; Sallaud et al., 2004). These banks are invaluable resources for establishing gene function in higher plants (Østergaard and Yanofsky, 2004).

To develop a resource for functional genomics of photosynthesis in Chlamydomonas, we have initiated a project to generate, screen, and obtain the flanking sequence from insertional mutants that exhibit photosynthesis-related phenotypes. This article details the phenotypic, molecular, and genetic characteristics of a subset of these mutants. Phenotypic analysis of the mutants confirmed that initial selection of transformants and subsequent maintenance of the mutants in the dark allows for the recovery of a large class of light-sensitive mutants (Table I), which might otherwise have been overlooked if nonphotosynthetic mutants were isolated by screening of light-grown cultures on minimal media (Spreitzer and Mets, 1981). Maintenance in the dark, however, may lead to the accumulation of light-sensitive, spontaneous mutations over time. This was found in the case of the CAL007.01.09 mutant (with an insertion in the carotene isomerase gene), which acquired an additional light-sensitive mutation that was revealed during genetic analysis. To minimize this problem, mutants are stored in liquid nitrogen or as a mated zygospore stock as soon as possible after isolation. RB and MZ were shown to be useful for the isolation of mutants that are sensitive to generators of reactive oxygen species. The choice of these two compounds was also found to be effective in differentiating the response to specific reactive oxygen species, as only one-third of the total number of RB- or MZ-sensitive mutants were found to be sensitive to both chemicals.

Molecular analysis of the mutant population showed that only approximately 30% of the mutants contained insertions of the *ble* gene at more than 1 locus, with an average number of insertions per clone of 1.4. This is comparable to Arabidopsis T-DNA mutant collections, in which the average number of T-DNA insertions per line is reported to be approximately 1.5 (McElver et al., 2001; Sessions et al., 2002; Alonso et al., 2003). Although a higher number of insertions per mutant means that fewer mutants are required to saturate the genome, isolation of the flanking sequence becomes more difficult when PCR-based techniques are used. In addition, the presence of numerous insertions per clone often has a negative impact on the mating ability of a clone and necessitates backcrossing to isolate the relevant mutation. It is therefore advantageous to maximize the number of clones with single inserts for both molecular and genetic reasons.

Genetic analysis showed that, in approximately 50% of the insertional mutants, the phenotype cosegregated with the transforming *ble* gene (Table III). This is in agreement with other insertional mutagenesis studies in Chlamydomonas (Niyogi et al., 1997; Fleischmann et al., 1999; Moseley et al., 2000). The tagging frequency in Chlamydomonas insertional mutagenesis therefore compares well with that reported for Arabidopsis T-DNA transformation, where as few as 35% of the mutants in a population may be tagged (McElver et al., 2001). It should also be noted that mutants in

Table IV. *TAIL-PCR cycling parameters used to isolate flanking DNA from insertional mutants*

Reaction	Step	Thermal Settings	No. of Cycles
Primary	1	95°C, 2 min	1
	2	94°C, 1 min; 62°C, 1 min; 72°C, 2.5 min	5
	3	94°C, 1 min; 25°C, 3 min; ramping to 72°C over 3 min; 72°C, 2.5 min	1
	4	94°C, 30 s; 68°C, 1 min; 72°C, 2.5 min; 94°C, 30 s; 68°C, 1 min; 72°C, 2.5 min; 94°C, 30 s; 44°C, 1 min; 72°C, 2.5 min	15
	5	72°C, 5 min	1
Secondary	1	94°C, 30 s; 64°C, 1 min; 72°C, 2.5 min; 94°C, 30 s; 64°C, 1 min; 72°C, 2.5 min; 94°C, 30 s; 44°C, 1 min; 72°C, 2.5 min	12
	2	72°C, 5 min	1
Tertiary	1	94°C, 30 s; 44°C, 1 min; 72°C, 2.5 min	20
	2	72°C, 5 min	1

which the screened photosynthesis-related phenotype is not tagged may be of interest in other fields of Chlamydomonas biology. For example, mutant CAL007.03.22 was found to contain an insertion adjacent to the gene encoding the 11-kD dynein light chain of the flagellar outer arm. It is unlikely that this would lead to the observed MZ- and RB-sensitive phenotype, but the mutant may have a linked motility phenotype that would not have been detected in our screening procedure.

Molecular analysis of the mutant population also revealed that, although all mutants analyzed had at least 1 copy of the *ble* gene, only approximately 50% of mutants had a band hybridizing to the origin of replication from the pBluescript region of the transforming plasmid. PCR screening also indicated that even fewer clones contained a fully intact origin of replication and ampicillin resistance gene (data not shown), suggesting that deletions affecting the transforming DNA occur frequently upon insertion into the Chlamydomonas genome. This illustrates why plasmid rescue has been a difficult technique to use in forward genetics studies in Chlamydomonas, as sequences required for the maintenance of the plasmid in *Escherichia coli* are frequently lost. In addition to the fact that plasmid rescue is not easily modified to higher throughput approaches, the above problem also explains why TAIL-PCR is the method that we have chosen for the isolation of the flanking sequence. Although PCR-based techniques are often difficult to optimize in Chlamydomonas due to the GC-rich nature and high occurrence of repeat regions in the genome, this article reports that TAIL-PCR was successful in amplifying fragments in almost 80% of the mutants analyzed. The only drawback of TAIL-PCR is that it cannot amplify through tandem arrays of inserts, and these occurred in approximately 15% to 20% of insertional mutants. This, however, compares favorably with Arabidopsis T-DNA mutant collections, in which 25% of left-border products and 62% of right-border products have been found to contain only T-DNA sequence (Sessions et al., 2002) using TAIL-PCR. Thus, the advantages of TAIL-PCR for higher throughput strategies outweigh its drawbacks.

Since the long-term aim of this project is to saturate the Chlamydomonas genome with mutations affecting photosynthesis, several other criteria in addition to insert number need to be examined. The number of insertional mutants required to saturate the genome is also dependent on the size of deletions that may occur at the site of insertion; larger deletions have the potential to affect multiple genes. Deletions of genomic DNA occurring at the point of insertion in Chlamydomonas range in size, but can be as large as 50 kb (Tanaka et al., 1998). The population described here appears to follow the same pattern. The mutant CAL007.01.15, for example, has a deletion of 36 kb (Dent et al., 2001), whereas CAL005.01.20 has only a few base pairs deleted at the site of insertion (data not shown). Calculations of the number of mutants needed also assume that insertion is a random event (Clarke and Carbon, 1976). Whether insertional mutagenesis is truly random in Chlamydomonas has also not been examined in previous studies. T-DNA insertion in Arabidopsis has been found to show bias against both predicted coding sequences and centromeres and to occur in preferred sites of integration or hot spots (Barakat et al., 2000; Sessions et al., 2002; Alonso et al., 2003). This work reports that, of the 50 flanking sequences isolated, 3 were found to be clustered within 50 kb of the *RBCS2* locus, and 2 were found associated with histone clusters (Table II). It is therefore possible that there is some site bias during insertional mutagenesis in Chlamydomonas, and this may be related to either sequence composition of the transforming DNA or variation in recombination frequency across the genome. It might be possible to minimize the impact of site bias in the mutant collection by using a variety of plasmids and selectable marker genes for insertional mutagenesis (Randolph-Anderson et al., 1998; Kovar et al., 2002; Depège et al., 2003). The issues of average deletion size and insertion site bias will need to be resolved once more mutants have been generated and characterized, thus allowing for a more accurate estimation of the number of mutant lines that need to be generated to achieve saturation.

Over the next several years, we aim to generate and screen 80,000 insertional mutant lines in Chlamydomonas. This will lead to the isolation of approximately 7,000 mutants affected in photosynthesis and sensitivity to photooxidative stress. Flanking sequences will be available as a searchable database within the Chlamydomonas Genome Project Web site (http://www.chlamy.org) and, when the final genome sequence is released, these sequences will be marked on the genome as an optional track within the browse function. Researchers can therefore either search the database with DNA sequences of interest or scan the genomic sequence surrounding their gene of interest for flanking sequence tags from mutants. The mutants will be available to the scientific community as mated zygospore stocks from the Chlamydomonas Genetics Center. Progeny recovered from heterozygous zygospores will represent a segregating population, which will allow for immediate genetic analysis of linkage between the mutant phenotype and the selectable marker used for transformation. Strains will also be stored frozen in liquid nitrogen to minimize the loss of mutants that are unable to mate. This population of mutants will represent the first publicly available catalogued collection of insertional mutants in Chlamydomonas, and it will be an invaluable resource for photosynthesis research.

MATERIALS AND METHODS

Media and Strains

Cultures of *Chlamydomonas reinhardtii* cells were grown heterotrophically or photoheterotrophically in Tris-acetate phosphate media (TAP) or

photoautotrophically in minimal high-salt (HS) media (Harris, 1989). Strain and mutant stocks were maintained on TAP agar medium in the dark at 25°C. For procedures that required liquid cultures, cells were grown in 50 mL TAP medium with shaking at 120 rpm either in the dark or at a very low light (VLL) intensity of 3 μmol photons m^{-2} s^{-1} at 25°C, except where otherwise stated.

The Chlamydomonas strain used to generate the population of mutants was selected for its ability to grow well and remain green in the dark on TAP medium. The growth of the standard laboratory strains CC125 (*mt*+) and CC124 (*mt*−), obtained from the Chlamydomonas Genetics Center (Duke University, Durham, NC), was compared with that of strains 4A+ and 17D−, which were obtained from J.-D. Rochaix (University of Geneva). Like CC125 and CC124, 4A+ (*mt*+) and 17D− (*mt*−) are in the 137c wild-type strain background.

For genetic analysis of the mutants generated in 4A+, a near-isogenic *mt*− strain (4A−), which showed similar sensitivity to HL and reactive oxygen species generators as 4A+, was generated by 4 backcrosses of 17D− to 4A+.

The strains used for the preparation of gamete autolysin were CC620 (137c NM subclone, *mt*+) and CC621 (137c NO subclone, *mt*−). These were also obtained from the Chlamydomonas Genetics Center.

Generation of Mutants and Genetic Crosses

Insertional mutagenesis of Chlamydomonas cells followed the transformation method of Kindle et al. (1989). One of 2 plasmids was used for transformation, pSP124S (Lumbreras et al., 1998) or pMS188 (Schroda et al., 2002), linearized with *Bam*HI or *Kpn*I, respectively (Fig. 1). Transformations with pSP124S used 1 μg plasmid DNA/transformation, whereas 0.6 μg of pMS188 were used. After transformation, the cells were allowed to recover in 10 mL TAP overnight in the dark at 25°C, with shaking at 110 rpm. The cells were then collected by centrifugation (1,300*g*, 3 min), resuspended in 300 μL TAP, and plated onto TAP agar plates containing 5 μg mL^{-1} zeocin (Invitrogen, Carlsbad, CA). The plates were maintained in the dark at 25°C for 3 to 4 weeks before the zeocin-resistant transformed colonies were picked.

Genetic crosses and tetrad analysis to assess linkage of the observed phenotype with antibiotic resistance were performed according to established methods (Harris, 1989).

Screening

Stock plates of insertional mutants were maintained in the dark on TAP agar plates. Prior to screening, the mutants were subcultured to fresh TAP plates and maintained at VLL at 25°C for 3 weeks. These VLL-acclimated mutants were used to inoculate 150 μL TAP in 96-well plates by replica plating. After 5 to 7 d of growth (VLL, 25°C), 3 μL cells were spotted onto each of the following primary screen plates: (1) TAP agar; (2) HS agar; (3) 1.5 mM MZ (Sigma, St. Louis) in TAP agar; and (4) 2 μM RB (Sigma) in TAP agar (Fig. 2). The TAP plates were maintained in the dark, the MZ and RB plates were incubated at a LL intensity of 80 μmol photons m^{-2} s^{-1}, and the HS plates were incubated at a HL intensity of 500 μmol photons m^{-2} s^{-1}. The temperature for all treatments was 25°C. All plates were scored for cell growth and bleaching after 7 to 10 d of treatment. Mutants displaying reduced growth or bleaching under any condition were picked for secondary screening.

For secondary screening, cells were grown and inoculated as described for the primary screens and submitted to 14 different treatments: (1) TAP agar (dark); (2) TAP agar (VLL); (3) TAP agar (LL); (4) HS agar (VLL); (5) HS agar (LL); (6) HS agar (HL); (7) HS agar (LL, with high CO_2); (8) HS agar (HL, with high CO_2); (9) 2 μM RB in TAP agar (VLL); (10) 2 μM RB in TAP agar (LL); (11) 1 μM RB in TAP agar (LL); (12) 1.5 mM MZ in TAP agar (VLL); (13) 1.5 mM MZ in TAP agar (LL); and (14) 1.5 mM MZ in HS agar (LL). High CO_2 atmosphere was achieved by incubating the plates in BBL GasPak CO_2 pouches (Becton-Dickinson, Franklin Lakes, NJ). In addition to these 14 treatments, the maximum chlorophyll fluorescence of the dark-grown TAP stock cultures was measured using video imaging (Polle et al., 2002).

DNA Extraction Techniques

Two different DNA extraction techniques were used in the study. For DNA required for Southern analysis, the extraction method followed that of Davies et al. (1992), excluding the final CsCl purification step. For DNA used for TAIL-PCR, cells were collected by centrifugation of 6-mL cell culture in TAP medium. The pellet was washed with 200 μL Milli-Q water, and DNA was extracted using DNAzol reagent (Invitrogen) according to the manufacturer's instructions. The final DNA pellet was resuspended in 100 μL Tris-EDTA (10 mM Tris, pH 8.0, 0.1 mM EDTA).

TAIL-PCR and Sequencing of Amplified Fragments

Genomic DNA adjacent to the insertion site of the transforming DNA was amplified using TAIL PCR (Liu et al., 1995). The method employed was optimized for Chlamydomonas. Flanking DNA was only isolated from the side of the insertion adjacent to the *ble* gene in each plasmid used, as it was found that random deletions of pBluescript sequences from the other end of the transforming DNA made amplification difficult. For pSP124S, the specific primers for primary, secondary, and tertiary reactions were RMD223 (5′-TTGGCTGCGCTCCTTCTGGCATTTAAATC-3′), RMD224 (5′-GCATTTAAATCTCGAGGTCGAC-3′), and RMD225 (5′-GATAAGCTTGATATCGAATTCC-3′), respectively. For pMS188, the specific primers for primary, secondary, and tertiary reactions were RMD264 (5′-GTGCTGAAGCGGTAGCTTAGCTCC-3′), RMD255 (5′-CTCCCCGTTTCGTGCTGATCAGTC-3′), and RMD256 (5′-GAGGAGTTTTGCAATTTTGTTGG-3′), respectively. Two arbitrary degenerate primers (Wu-Scharf et al., 2000) were tested for amplification, RMD227 (5′-NTCGWGWTSCNAGC-3′) and RMD228 (5′-WGNTCWGNCANGCG-3′). RMD227 was found to amplify flanking regions successfully in most samples, whereas RMD228 only resulted in fragments in 50% of samples tested. RMD227 was therefore selected as the degenerate primer for all future reactions.

Primary TAIL-PCR reactions (20 μL) contained 1× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$), 200 μM of each dNTP, 5 pmol RMD223 or RMD264, depending on the plasmid used for transformation, 60 pmol RMD227, and 2.5 units Taq polymerase (Eppendorf AG, Hamburg, Germany). The cycling parameters for all reactions of TAIL-PCR are described in Table IV.

Primary reactions were diluted 25-fold and 2-μL aliquots added directly to secondary TAIL-PCR reactions (20 μL), which contained identical components and concentrations to the primary reaction with the exception that the specific primer was replaced with RMD224 or RMD255. For the low-stringency tertiary reaction, the secondary reaction was again diluted 25-fold and either 1- or 2-μL aliquots, depending on the level of amplification achieved at the secondary stage, were added to the tertiary reaction (50 μL). Again these components were identical to that of the primary reaction, using the specific primers RMD225 or RMD256. The amplified products from both the primary and secondary reactions were analyzed by agarose gel electrophoresis. Reactions were purified as follows prior to sequencing. For samples where a single band was amplified, DNA from the tertiary PCR reaction mix was isolated using the QIAquick PCR purification kit (Qiagen, Valencia, CA). If more than one band was amplified, the fragments were separated by agarose gel electrophoresis, and individual fragments were isolated from the gel using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions.

For direct sequencing, 10 to 60 ng DNA were amplified with 10 pmol/reaction of RMD225 (pSP124S) or RMD256 (pMS188) using the DYEnamic ET Terminator Cycle Sequencing kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions, including the optional dilution buffer at a 1:1 (v/v) dilution. Sequencing reactions were run on an ABI3100 sequencer. Sequence data are available at the Chlamydomonas Genome Project Web site (http://www.chlamy.org).

Zygospore Storage and Cryopreservation of Cells

All mutants that showed a phenotype after the secondary round of screening were stored as both zygospores and frozen cells. For zygospore storage, each mutant was mated to the strain 4A−, and the mating mix was added to clay particles (unscented cat litter) and allowed to dry as described (Harris, 1989). Cryopreservation of cultures was carried out as described previously (Crutchfield et al., 1999).

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

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